Identification of proteins involved in formaldehyde metabolism by *Rhodobacter sphaeroides*

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Formaldehyde is an intermediate formed during the metabolism of methanol or other methylated compounds. Many Gram-negative bacteria generate formaldehyde from methanol via a periplasmic pyrroloquinoline quinone (PQQ)-dependent dehydrogenase in which the $\alpha$ subunit of an $\alpha_2\beta_2$ tetramer has catalytic activity. The genome of the facultative formaldehyde-oxidizing bacterium *Rhodobacter sphaeroides* encodes XoxF, a homologue of the catalytic subunit of a proposed PQQ-containing dehydrogenase of *Paracoccus denitrificans*. *R. sphaeroides* xoxF is part of a gene cluster that encodes periplasmic c-type cytochromes, including CycI, isocytochrome $c_2$ and CycB (a cyt $c_{553}$ homologue), as well as adhl, a glutathione-dependent formaldehyde dehydrogenase (GSH-FDH), and gfa, a homologue of a glutathione–formaldehyde activating enzyme (Gfa). To test the roles of XoxF, CycB and Gfa in formaldehyde metabolism by *R. sphaeroides*, we monitored photosynthetic growth with methanol as a sole source of carbon. Our data show that *R. sphaeroides* cells lacking XoxF or CycB do not exhibit methanol-dependent oxygen uptake and lack the capacity to utilize methanol as a sole photosynthetic carbon source. These results suggest that both proteins are required for formaldehyde metabolism. *R. sphaeroides* Gfa is not essential to activate formaldehyde, as cells lacking gfa are capable of both methanol-dependent oxygen uptake and growth with methanol as a photosynthetic carbon source.

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**INTRODUCTION**

Formaldehyde is produced at significant levels by abiotic and biological processes (Auerbach *et al.*, 1977; Levy, 1971). Because this compound can inactivate many cellular components, cells have systems to metabolize formaldehyde (Auerbach *et al.*, 1977; Barber & Donohue, 1998). We are studying proteins involved in formaldehyde metabolism by *Rhodobacter sphaeroides*, a bacterium with an inducible system for the metabolism of this compound (Barber & Donohue, 1998; Hickman *et al.*, 2004).

The study of formaldehyde metabolism in purple nonsulfur photosynthetic bacteria such as *R. sphaeroides* began when Quayle and co-workers isolated species capable of photosynthetic growth on medium containing methanol and bicarbonate (Quayle & Pfennig, 1975; Sahm *et al.*, 1976). In media supplemented with methanol or methylated compounds such as choline, *R. sphaeroides* utilizes a GSH-dependent pathway to metabolize formaldehyde (Barber *et al.*, 1996; Barber & Donohue, 1998). Subsequent studies have shown that *R. sphaeroides* is not able to grow on methylamine (Barber *et al.*, 1996; Barber & Donohue, 1998), and that the genome of this bacterium lacks genes needed for the metabolism of this and other one-carbon ($C_1$) methyl donors (Mackenzie *et al.*, 2001).

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In *R. sphaeroides*, formaldehyde (CH$_2$O) is added to the thiol of glutathione to form $\text{S-hydroxymethylGSH}$ (GS-CHO), a reaction which can either occur spontaneously or be facilitated by a glutathione/formaldehyde-activating enzyme (Gfa) (Goenrich *et al.*, 2002). GS-CH$_2$OH is oxidized to $\text{S-formylGSH}$ (GS-CHO) by a GSH-dependent...

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**Abbreviations:** Ap, ampicillin; cyt, cytochrome; GS-CH$_2$OH, S-hydroxymethylGSH; GSH-FDH, glutathione-dependent formaldehyde dehydrogenase; isocyt, isocytochrome; PQQ, pyrroloquinoline quinone; RC, reaction centre; Tc, tetracycline; Tp, trimethoprim.

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formaldehyde dehydrogenase (GSH-FDH), then converted to formate (HCOO\(^{-}\)) by S-formylGSH hydrolase (FGH). Finally, formate is oxidized by formate dehydrogenase (FDH) to carbon dioxide (CO\(_{2}\)). The \textit{R. sphaeroides} GSH-FDH structural gene (adhi) is part of a potential formaldehyde metabolism gene cluster (gfa-adhi-cycl-xoxF-cycB-RSP2580-RSP2581) (Mackenzie et al., 2001) that encodes proteins with high amino acid identity to those involved in C\(_{1}\) metabolism by facultative methylotrophs (Fig. 1a) (Chistoserdova, 1996; Harms & van Spanning, 1991; Harms et al., 1996; Ras et al., 1991). In contrast, \textit{R. sphaeroides} genes encoding FGH and FDH homologues map elsewhere in the genome (Mackenzie et al., 2001).

The periplasmic oxidation of methanol to formaldehyde is often catalysed by proteins that contain pyrroloquinoline quinone (PQQ) as a prosthetic group (Anthony & Williams, 2003; Goodwin & Anthony, 1998). In such PQQ-dependent methanol dehydrogenases, the \(\alpha\) subunit (MxaF) has catalytic activity while the role of the \(\beta\) subunit (MxaI) is not known (Anthony & Williams, 2003; Goodwin & Anthony, 1998). \textit{R. sphaeroides} XoxF has amino acid sequence similarity to MxaF and XoxF of \textit{Paracoccus denitrificans}. \textit{P. denitrificans} XoxF has been implicated in formaldehyde formation from methanol, but an XoxF mutant of this bacterium is able to grow with methanol as a sole carbon source, albeit to a reduced extent, presumably because cells also contain MxaI (Harms et al., 1996; Ras et al., 1991). In \textit{Methylbacterium extorquens}, the reduction of PQQ is followed by successive one-electron transfers to periplasmic \(c\)-type cytochromes which bring reducing power to membrane-bound proteins (Anthony, 1992; Goodwin & Anthony, 1998).

This work takes advantage of features of \textit{R. sphaeroides} to investigate proteins needed for formaldehyde metabolism. \textit{R. sphaeroides} is able to co-metabolize methanol and other sources of formaldehyde to CO\(_{2}\) in both the presence and the absence of oxygen. \textit{R. sphaeroides} does not assimilate the CO\(_{2}\) produced during aerobic respiration, as Calvin cycle enzyme activity is not present (Barber & Donohue, 1998). Under photosynthetic conditions (anaerobic plus

![Fig. 1. Succinate and methanol oxidation by \textit{R. sphaeroides}. (a) Shown is the direction of gene transcription (arrowhead) and the intergenic distances within this region of the \textit{R. sphaeroides} genome. Trimethoprim (Tp\(^{+}\)) cartridge insertion is indicated by \(\Delta\); the extent of wild-type DNA in cosmid pUI8747 is shown above the map. Known operons are shown by a dashed arrow (Barber et al., 1996; Rott et al., 1993), whereas dotted arrows show potential operons (Y. Dufour, personal communication). (b) Periplasmic oxidation of methanol to formaldehyde results in the concomitant transfer of electrons to CycB and to isocyt \(c_{2}\), and the reduction of O\(_{2}\) by a terminal cytochrome oxidase. Alternatively, under photosynthetic conditions, electrons can be transferred to RC complexes in the membrane. (c) During succinate-dependent respiration, succinate dehydrogenase (SDH) reduces ubiquinone (UQH\(_{2}\)) via a terminal quinol oxidase. Alternatively, electrons from quinone can pass through cyt bc\(_{1}\), then to periplasmic cytochromes such as isocyt \(c_{2}\), and finally onto membrane-bound cytochrome oxidase to reduce O\(_{2}\).](http://mic.sgmjournals.org)
light), *R. sphaeroides* can use methanol as a sole carbon source because it assimilates CO₂ via the Calvin cycle (Barber & Donohue, 1998). Since the *R. sphaeroides* genome lacks mxaFI (MxaFI), the contribution of XoxF to formaldehyde metabolism can be easily studied. In addition, *R. sphaeroides* encodes isocytrome (isocyt) c₂ and CycB, a homologue of soluble cyt c proteins of *M. extorquens* (MxaG') and *P. denitrificans* (cyt c₅₅₃), which could carry electrons from a periplasmic dehydrogenase to the membrane (Anthony, 1992; Harms & van Spanning, 1991; Ras et al., 1991; Rott et al., 1992, 1993). Furthermore, *R. sphaeroides* Gfa is a homologue of a *P. denitrificans* protein (Goenrich et al., 2002b; Neculai et al., 2005) which forms the GSH-FDH substrate (Barber et al., 1996; Barber & Donohue, 1998). To analyse the requirements for formaldehyde metabolism by *R. sphaeroides*, we used growth in the presence of methanol and whole-cell O₂ uptake with wild-type cells as well as mutants lacking XoxF, PQQ, one or more c-type cytochromes, or Gfa.

### METHODS

**Bacterial strains and growth conditions.** *R. sphaeroides* strains (Table 1) were grown in Sistrom’s minimal medium (Sistrom, 1960) containing 35 mM succinate at 30 °C. For testing aerobic methanol metabolism, the medium was supplemented with 100 mM methanol. Monitoring photosynthetic growth by *R. sphaeroides* utilized either a succinate-based medium or one lacking succinate and amino acids but containing 100 mM methanol and 40 mM sodium bicarbonate. *Escherichia coli* strains were grown at 37 °C in Luria–Bertani medium. *E. coli* strain DH5α was used as a plasmid host and S17-1 was used for conjugation of plasmids into *R. sphaeroides* (Davis et al., 1988). When necessary, the medium was supplemented with 30 μg ml⁻¹ trimethoprim (Tp) or 1 μg ml⁻¹ tetracycline (Tc) for *R. sphaeroides*, and 100 μg ml⁻¹ ampicillin (Ap), 10 μg Tc ml⁻¹ or 100 μg Tp ml⁻¹ for *E. coli*.

### Table 1. Plasmids and strains

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<th>Plasmid or strain</th>
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<td>Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Simon (1979)</td>
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<td>pSUP202</td>
<td>Ap&lt;sup&gt;R&lt;/sup&gt;, Tc&lt;sup&gt;R&lt;/sup&gt;, Km&lt;sup&gt;R&lt;/sup&gt;, Mob&lt;sup&gt;+&lt;/sup&gt;; pBR322 derivative</td>
<td>Simon et al. (1983)</td>
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<tr>
<td>pUC19</td>
<td>Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Wasson et al. (1979)</td>
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<tr>
<td>pSUP202</td>
<td>Ap&lt;sup&gt;R&lt;/sup&gt;, Tc&lt;sup&gt;R&lt;/sup&gt;, Km&lt;sup&gt;R&lt;/sup&gt;, Mob&lt;sup&gt;+&lt;/sup&gt;; pBR322 derivative</td>
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<td>pUC19</td>
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<td><strong>Strains</strong></td>
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<td>E. coli</td>
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<tr>
<td>DH5α</td>
<td>supE44 ΔlacU169 (Φ80 lacZΔM15) hsdR178 recA1 endA1 gyrA96 thi-1 relA1</td>
<td>Bethesda Laboratories Research (1986)</td>
</tr>
<tr>
<td>S17-1</td>
<td>C600::RP-4 2–(Tc::Mu) (Kn::Tn7 thi pro hsdR hsdM&lt;sup&gt;+&lt;/sup&gt; recA)</td>
<td>Simon et al. (1983)</td>
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Molecular methods and plasmid construction. Standard methods were used for preparation of plasmid DNA from E. coli. Modification enzymes, restriction endonucleases and Taq or Pfu polymerase were used as instructed by the manufacturers. Genomic DNA from wild-type and mutant strains of R. sphaeroides was isolated (Chen & Kuo, 1993) and amplified with Pfu polymerase to facilitate cloning into EcoRV-digested Litusm 281. Candidate genes were analysed by DNA sequence analysis using the Big Dye Cycle Sequencing protocol (Perkin Elmer). Reaction products were applied to Sephadex G-50 columns and analysed at the University of Wisconsin-Madison DNA Sequencing Facility. Primer sequences used are available from the authors upon request.

Creation of R. sphaeroides mutants. Primers specific to the xoxF upstream and downstream regions, both containing an EcoRI restriction endonuclease site, were used to amplify DNA from pWT-P1. These restriction endonuclease sites facilitated cloning of the 1958 bp PCR product in EcoRI-digested pUC19 (pXoxF2). Divergent primers were used with pXoxF2 to create a 589 bp deletion flanked by 780 bp of xoxF upstream DNA and 520 bp downstream DNA in pUC19. To degrade parental DNA, DpnI was added to the PCR product and incubated for 6 h at 37 °C. The 34S-Tp Smal-digested Tpβ cartridge (Dennis & Zylstra, 1998) was ligated into the pXoxF2 PCR product and transformed into E. coli DH5α (pTP16). The EcoRI insert of pTP16 was isolated and cloned into EcoRI-digested pSUP202 (pTP16-12). After mobilization of pTP16-12 into R. sphaeroides, Tpα isolates were screened for TcR, to monitor the recombination of ΔoxoxF::dhfRII by an even number of crossover events (strain TP19).

Upstream and downstream cycB primers with PsI restriction endonuclease sites were used to amplify DNA from pWT-P1. The 1210 bp PCR product contained an internal EcoRI site producing a PsI-EcoRI fragment that was cloned into a similarly digested pUC19 (pSW502). Divergent primers with XbaI restriction endonuclease sites were used with pSW502, creating a 120 bp deletion with 524 and 533 bp of upstream and downstream sequence, respectively, in pUC19. This PCR product was treated with DpnI. Following digestion of p34S-Tp with XbaI, a Tpβ cartridge was inserted into pSW502 (digested with XbaI) and transformed into DH5α (pSW517). The EcoRI-PsI cycB-containing fragment of pSW517 was cloned into pSUP202 to produce pSW521. Following conjugal transfer of pSW521 into R. sphaeroides, strains exhibiting a Tpβ TcC phenotype were analysed to confirm cycB::dhfRII gene incorporation into the genome by an even number of crossover events (strain TP19).

A 1.2 kb region that included gfa was amplified with primers containing EcoRI sites and cloned into EcoRI-digested Litusm 281 (pSW161). Using divergent primers with Sphl restriction sites, a 147 bp deletion was created in pSW161 and the PCR product was treated with DpnI. Following Sphl endonuclease treatment of p34S-Tp, the Tpβ cartridge was ligated into pSW161 (pSW161-4). The gfa-containing EcoRI fragment of pSW161-4 was cloned into pSUP202 to produce pSW170. After mobilization of pSW170 into R. sphaeroides, Tpβ strains were screened for TcC to score recombination of the Δgfa::dhfRII allele into the genome by an even number of crossover events.

Cosmid pUI8747 (Rott et al., 1993), which contains wild-type R. sphaeroides DNA, was mobilized into TP19 (XoxF mutant) or SW531 (CycB mutant) to test for complementation.

Photosynthetic methanol utilization. To test for photosynthetic methanol utilization, aerobically grown cells were harvested and washed with Sistrom’s medium lacking a carbon source. Following dilution into Sistrom’s succinate media (~1.0 x 10^8 c.f.u. ml^-1), cells were placed in filled screw-capped test tubes and exposed to an incandescent light source (10 W m^-2); measured with a Yellow-Springs-Kettering model 6.5-A radiometer). After 3 days, cells were harvested, washed twice in media lacking a carbon source and diluted (~1.0 x 10^8 c.f.u. ml^-1) in media containing 100 mM methanol and 40 mM sodium bicarbonate. These tubes were exposed to light and monitored for up to 7 days to score photosynthetic growth.

Whole-cell O2 uptake. Whole-cell O2 uptake was monitored using a YSI model 5300A meter equipped with a Clark-type polarographic O2 probe. For these assays, aerobically grown cultures (~2.5 x 10^8 c.f.u. ml^-1) were exposed to 100 mM methanol for 3 h to induce expression of genes required for methanol metabolism (Hickman et al., 2004). These cells were harvested by centrifugation (10 min at 5000 g) and washed twice in medium lacking succinate. Then, ~5.0 x 10^9 c.f.u. ml^-1 of washed cells were added to the sample chamber for O2-uptake assays. O2-consumption rates were determined in the absence of added carbon sources (endogenous rate), in the presence of 35 mM succinate (succinate-dependent respiration) or in the presence of 25–100 mM methanol (methanol-dependent rate). For each assay, measurements were recorded every 15 s over a 240 s period. By plotting O2 consumption as a function of time, an endogenous, succinate-, or methanol-dependent rate of O2 uptake was calculated [nmol O2 consumed min^-1 (mg protein)^-1].

Computational prediction of potential operons. To predict co-transcribed ORFs (Fig. 1a), Pearson’s correlation coefficients were calculated based on RNA abundance measurements from public-domain R. sphaeroides global gene expression datasets (Gene Expression Omnibus; http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GPL162) (Y. Dufour, personal communication). Statistical probabilities for co-transcription of ORFs were obtained from available models (www.microbesonline.org) (Bockhorst et al., 2003; Price et al., 2005).
RSP2579 (64%) with the mono-haem cyt C336 (cytB) of P. denitrificans (Harms et al., 1996; Ras et al., 1991), we have designated this ORF as R. sphaeroides CycB (predicted pI of 4.2). A haem-staining protein of a molecular mass predicted for the mature CycB protein (~18 kDa after cleavage of predicted signal peptide) has been observed previously in R. sphaeroides Spd mutants, which have increased expression of some genes in this cluster (Rott et al., 1992). RSP2580 has 63% amino acid identity to XoxJ of P. denitrificans (Harms et al., 1996) and resembles bacterial periplasmic solute binding proteins (Saier, 1994, 2000). Finally, RSP2581 has 62% amino acid sequence identity to P. denitrificans XoxI. RSP2581 is predicted to have a rhodanese-like fold (Bordo & Bork, 2002; Spallarossa et al., 2003, 2004), a pI of ~4.8, and a molecular mass of 19 kDa (www.expasy.org). While similar clusters of related genes are found in genomes from several bacteria, the contribution of xoxF-cycB-RSP2580-RSP2581 to formaldehyde metabolism remains unclear. To test the role of these gene products in formaldehyde metabolism, we used enzymic and phenotypic assays to monitor the function of this pathway in wild-type and mutant cells.

Requirements for R. sphaeroides formaldehyde metabolism

One test of R. sphaeroides formaldehyde metabolism was a whole-cell O2-uptake assay. We used this assay because earlier attempts to monitor methanol oxidation by other methods in cell extracts of purple non-sulfur bacteria have been unsuccessful for unknown reasons (Hickman, 2003; Quayle & Pfennig, 1975; Sahm et al., 1976).

To enhance our ability to assay methanol-dependent O2 uptake, cells were grown in the presence of methanol for one doubling prior to analysis. These growth conditions are known to increase the expression of genes required for the metabolism of this carbon source (Hickman et al., 2004) and expression of genes involved in formaldehyde oxidation (Barber & Donohue, 1998). This regimen was preferred over growing cells in the presence of methanol for longer periods because we intended to assay both wild-type and mutant cells that might have contained defects in the formation or metabolism of formaldehyde.

Following exposure to methanol, cells were harvested and suspended in medium lacking any carbon source to allow determination of a background (endogenous) O2-uptake rate. O2-uptake activity was then monitored after the addition of methanol or succinate (Fig. 2). The endogenous rate of O2 uptake (O2 consumed per unit time) was subtracted from the rate determined in the presence of succinate or methanol (Fig. 2).

R. sphaeroides Ga was used as the parent strain because many of the mutants previously analysed for defects in formaldehyde metabolism have been generated in this background (Barber, 1997; Hickman et al., 2002; Ozcan, 1996; Rott et al., 1993). After subtracting the endogenous rate of O2 uptake, a significant and reproducible rate of methanol-dependent O2 uptake was measured in strain Ga (~5.0 nmol O2 min\(^{-1}\) (mg protein)\(^{-1}\); Table 2). For comparison, the rate of whole-cell O2 uptake was ~15.8 nmol O2 min\(^{-1}\) (mg protein)\(^{-1}\) when succinate was provided as the electron donor (Table 2). These data indicate that the whole-cell O2 uptake assay is able to report on the use of methanol or succinate as an electron donor. It is not surprising that the rates of O2 uptake are higher with succinate as an electron donor than with methanol, since it is known that wild-type cells have a shorter doubling time in minimal medium that contain

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**Fig. 2.** Whole-cell O2 uptake by wild-type cells. Plotted is the percentage O2 concentration as a function of time for each carbon source tested. (•) Data used to calculate the endogenous rate of O2 uptake (O2 consumption without the addition of any carbon source); (■) samples containing methanol; (▲) samples containing succinate.
this compound as a sole carbon source (Barber et al., 1996; Barber & Donohue, 1998).

To further verify the utility of this assay, we monitored whole-cell O2 uptake in mutants with known or predicted defects in formaldehyde metabolism. Based on what is known about the formaldehyde metabolism of other Gram-negative bacteria, we predict that *R. sphaeroides* would require a PQQ-containing enzyme to generate formaldehyde from methanol. To test this hypothesis, BOX9, a mutant with a loss-of-function mutation in PQQ synthesis, was assayed for methanol oxidation by the whole-cell O2-uptake assay. The rate of O2 consumption in the presence of succinate by *R. sphaeroides* BOX9 was comparable to that of wild-type cells (Table 2). This was not surprising, since PQQ is not required for electron transport from succinate to O2 (Fig. 1c). In contrast, when methanol was used as an electron donor there was no detectable O2 consumption in the BOX9 strain over background. Results of control experiments indicate that cells lacking AdhI have a rate of methanol-dependent O2 uptake comparable to that of wild-type cells (data not shown). Thus, the deficiency in methanol-dependent O2 uptake exhibited by BOX9 demonstrates the requirement for the PQQ prosthetic group for the conversion of methanol to formaldehyde by *R. sphaeroides* (Table 2).

Methanol metabolism by Gram-negative bacteria often involves periplasmic c-type cytochromes to transfer electrons from a PQQ-dependent dehydrogenase to membrane-bound enzymes of the respiratory chain (Anthony, 1982, 1992) (Fig. 1b). As predicted, in CCMA1 cells, which contain a mutation that blocks assembly of many periplasmic and membrane-associated cyt c proteins, there is no detectable methanol-dependent O2 uptake compared to background (Table 2). In contrast, succinate-dependent O2 uptake is not abolished in CCMA1 cells (Table 2), presumably because *R. sphaeroides* contains a quinol oxidase which allows cyt c-independent electron transfer from succinate via quinol to O2 (Fig. 1c). These results demonstrate that methanol-dependent respiration in *R. sphaeroides* requires one or more cyt c proteins, presumably to transfer electrons from a periplasmic PQQ-dependent enzyme to O2. Indeed, a reduced methanol-dependent O2-uptake rate [~2.2 nmol O2 min⁻¹ (mg protein)⁻¹] is observed in strain CYCI1 (lacks CycI; Table 2), suggesting a potential role for isocyt c2 in electron transfer during formaldehyde generation by this periplasmic protein. On the other hand, no detectable methanol-dependent O2 uptake was measured in CYCA1, which has mutations that inactivate both cycA and the gene that encodes its isoform, cycI (Table 2).

### Additional proteins required for *R. sphaeroides*

Using the O2 uptake assay, we also analysed the properties of *R. sphaeroides* cells containing defined mutations in *xoxF*, *cycB* or *gfa* (Fig. 1a). Based on the direction of transcription of *xoxF* and *gfa* as well as their predicted positions in potential operons, we do not expect the properties of these mutants to be complicated by polarity on downstream genes (Fig. 1a). Methanol-dependent O2 uptake of *R. sphaeroides* cells that lacked XoxF (TP19) was not detectable over background (Table 3). As expected, cells lacking XoxF exhibited normal rates of O2 uptake when using succinate as an electron donor (Table 3). Also, the ability to complement the defect of TP19 with cosmid pUI8747 (strain SW426) demonstrates that XoxF is required for methanol-dependent O2 uptake by *R. sphaeroides* (Table 3). Additionally, cells that lacked CycB (SW531) were incapable of methanol-dependent O2 consumption (Table 3). When SW531 cells containing pUI8747 (strain SW538) were analysed, rates of methanol-dependent O2 uptake were restored (Table 3) to levels observed in wild-type cells. This suggests that CycB is also required for methanol-dependent O2 uptake by *R. sphaeroides*, presumably since it carries electrons from the enzyme that generates formaldehyde to the respiratory chain (Fig. 1b). Thus, it appears that inhibiting electron transfer from the site of formaldehyde formation is sufficient to prevent methanol-dependent O2 uptake. In the case of both TP19 and SW531, the addition of ascorbate was able to support O2 uptake (data not shown). Ascorbate donates electrons directly to cytochrome oxidases; therefore, both of these strains contain a functional terminal cytochrome oxidase(s) (Fig. 1b).

### Table 2. Methanol metabolism in PQQ and c-type cyt mutants

<table>
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<th>Strain</th>
<th>Relevant genotype</th>
<th>O2 uptake*</th>
<th>PS growth†</th>
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<tr>
<td></td>
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<td>Succinate</td>
<td>MeOH</td>
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<td>Ga</td>
<td><em>ctrD</em></td>
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<td>2.2 ± 0.3</td>
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<td><em>cycA cycI::Ω</em></td>
<td>14.1 ± 1.0</td>
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*Values shown for O2 consumption have been corrected by subtracting the endogenous rate of O2 uptake [~2 nmol O2 (mg protein)⁻¹]. †Photosynthetic (PS) growth of bacteria in liquid medium with succinate, succinate and methanol, or only methanol; (+) growth, (−) no growth.

Formaldehyde metabolism by *R. sphaeroides*

Using the O2-uptake assay, we also analysed the properties of *R. sphaeroides* cells containing defined mutations in *xoxF*, *cycB* or *gfa* (Fig. 1a). Based on the direction of transcription of *xoxF* and *gfa* as well as their predicted positions in potential operons, we do not expect the properties of these mutants to be complicated by polarity on downstream genes (Fig. 1a). Methanol-dependent O2 uptake of *R. sphaeroides* cells that lacked XoxF (TP19) was not detectable over background (Table 3). As expected, cells lacking XoxF exhibited normal rates of O2 uptake when using succinate as an electron donor (Table 3). Also, the ability to complement the defect of TP19 with cosmid pUI8747 (strain SW426) demonstrates that XoxF is required for methanol-dependent O2 uptake by *R. sphaeroides* (Table 3). Additionally, cells that lacked CycB (SW531) were incapable of methanol-dependent O2 consumption (Table 3). When SW531 cells containing pUI8747 (strain SW538) were analysed, rates of methanol-dependent O2 uptake were restored (Table 3) to levels observed in wild-type cells. This suggests that CycB is also required for methanol-dependent O2 uptake by *R. sphaeroides*, presumably since it carries electrons from the enzyme that generates formaldehyde to the respiratory chain (Fig. 1b). Thus, it appears that inhibiting electron transfer from the site of formaldehyde formation is sufficient to prevent methanol-dependent O2 uptake. In the case of both TP19 and SW531, the addition of ascorbate was able to support O2 uptake (data not shown). Ascorbate donates electrons directly to cytochrome oxidases; therefore, both of these strains contain a functional terminal cytochrome oxidase(s) (Fig. 1b). Thus, the failure of TP19 and SW531 to exhibit detectable
methanol-dependent O$_2$ uptake is not due to lack of this terminal enzyme in the respiratory chain.

To analyse the role of *R. sphaeroides* Gfa in formaldehyde metabolism (Goenrich *et al.*, 2002) we tested O$_2$ uptake by cells that lacked Gfa (SW102). Cells lacking Gfa (SW102) were capable of using succinate or methanol as an electron donor to support O$_2$ uptake; however, the rate of methanol-dependent O$_2$ uptake was slightly lower than that of wild-type cells (Table 3). Based on the observed activity of SW102, we conclude that loss of Gfa is not sufficient to prevent methanol-dependent O$_2$ uptake by *R. sphaeroides*.

**Table 3. Properties of methanol metabolism mutants**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>O$_2$ uptake*</th>
<th>PS growth†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Succinate</td>
<td>MeOH</td>
</tr>
<tr>
<td>Ga</td>
<td><em>crtD</em></td>
<td>15.8±1.0</td>
<td>5.0±0.7</td>
</tr>
<tr>
<td>TP19</td>
<td><em>crtD ΔxoxF::Tp</em></td>
<td>21.5±1.0</td>
<td>≤0.2</td>
</tr>
<tr>
<td>SW426</td>
<td><em>crtD TP19 with cosmid pUI8747</em></td>
<td>12.6±0.5</td>
<td>6.2±1.0</td>
</tr>
<tr>
<td>SW531</td>
<td><em>ΔcycB::Tp</em></td>
<td>16.1±2.0</td>
<td>≤0.2</td>
</tr>
<tr>
<td>SW538</td>
<td><em>crtD SW531 with cosmid pUI8747</em></td>
<td>13.0±0.5</td>
<td>6.6±0.7</td>
</tr>
<tr>
<td>SW102</td>
<td><em>Δgfa::Tp</em></td>
<td>18.0±1.4</td>
<td>3.6±0.9</td>
</tr>
</tbody>
</table>

*Values shown for O$_2$ consumption have been corrected by subtracting the endogenous rate of O$_2$ uptake (~2 nmol O$_2$ min$^{-1}$ (mg protein)$^{-1}$).
†Photosynthetic (PS) growth of bacteria in liquid medium with succinate, succinate and methanol, or only methanol; (+) growth, (–) no growth.

**DISCUSSION**

In this work, we analysed products from a region of the *R. sphaeroides* genome, *gfa-adhI-cycl-xoxF-cycB-RSP2580-RSP2581*, which encodes proteins implicated in the metabolism of formaldehyde (Fig. 1). To address the function of these gene products in *R. sphaeroides*, mutants lacking the PQQ-dependent dehydrogenase (XoxF), a homologue of the periplasmic cyt $c_533$ (CycB) or the glutathione formaldehyde activating enzyme (Gfa), were monitored for methanol-dependent O$_2$ uptake or photosynthetic growth using methanol as a carbon source (Table 3). We used methanol as a carbon source for these studies, as previous work has shown that growth under these conditions requires the photosynthetic growth for both the PQQ and XoxF mutants is a consequence of the requirement for a PQQ-dependent enzyme such as XoxF. In addition, the CycB mutant (SW531) was able to grow photosynthetically in the presence of succinate but not when methanol was used as the sole carbon source (Table 3). Based on this we predict that CycB is needed to bring electrons from the PQQ-dependent dehydrogenase XoxF to the photosynthetic electron transport chain (Fig. 1b). As expected, the photosynthetic growth defects in the presence of methanol of the XoxF and CycB mutants were each complemented by a cosmid (pUI8747, Fig. 1a) that carries this entire region of the genome (Table 3). Finally, photosynthetic growth of cells that lack XoxF, CycB or PQQ occurred when the media contained both succinate and methanol (Tables 2 and 3), indicating that these mutants are not simply sensitive to methanol. Rather, it appears that the loss of PQQ, XoxF or CycB causes the growth defect by blocking the pathway needed to generate formaldehyde from methanol under photosynthetic conditions.

In contrast, cells lacking Gfa (SW102) grew photosynthetically when using either succinate or methanol (Table 3). These data, plus the ability to measure methanol-dependent O$_2$ uptake in SW102 cells, support the notion that Gfa is not absolutely required for formaldehyde activation and its subsequent metabolism by GSH-FDH in *R. sphaeroides*.**
activity of GSH-FDH to metabolize the formaldehyde that is produced (Barber et al., 1996; Hickman et al., 2002). Also, by analysing strains that lacked periplasmic c-type cytochromes (CYCI1, CYCAI and CCMA1) or possessed a defect in PQ synthesis, we were able to study other requirements for formaldehyde metabolism (Table 2). Our results show that formaldehyde generation from methanol by R. sphaeroides requires XoxF, PQ and periplasmic c-type cytochromes, including CycB. However, R. sphaeroides does not require Gfa for the metabolism of formaldehyde.

Whole-cell methanol-dependent O₂ uptake and the use of methanol as a sole photosynthetic carbon source require either XoxF or PQ biosynthesis (Table 3). Because R. sphaeroides BOX9 contains a lesion in PqqE, this finding suggests that XoxF requires PQ for formaldehyde metabolism (Ozcan, 1996). The properties of the R. sphaeroides XoxF mutant (TP19) and the absence of a gene related to mxaF which interacts with MxaI (Ghosh et al., 1995). This also suggests that active XoxF might not need a b subunit to form an active enzyme. Phylogenetic and bioinformatic analysis found high amino acid identity among other XoxF homologues (Fig. 3), even though the genomes of P. denitrificans, M. extorquens and Rhodopseudomonas palustris BisA53 contain both mxaFl and a homologue of xoxF.

Interestingly, R. sphaeroides lacks detectable activity using the dye-linked assay that is commonly employed with a₂b₂ methanol dehydrogenases (Fig. 3) (Hickman, 2003; Quayle & Pfennig, 1975; Sahm et al., 1976). The inability to detect dye-linked methanol dehydrogenase activity in extracts may be due to the absence of a b subunit which can stabilize the enzyme (Ghosh et al., 1995).

It is accepted that periplasmic c-type cytochromes are involved in electron transfer to the membrane following the oxidation of methanol (Anthony, 1992). Since R. sphaeroides possesses well-characterized periplasmic electron transport carriers, we also tested the role of these proteins in methanol metabolism. One ORF in the proposed R. sphaeroides formaldehyde metabolism gene cluster encodes a c-type cyt with high amino acid identity to cyt c₅₅₃ and mutational analysis of cycB showed that the protein is required for growth and O₂ uptake when R. sphaeroides is placed in formaldehyde-generating conditions. Although the genomes of P. denitrificans or M. extorquens (MxaG') have been suggested to be involved in C₁ metabolism, earlier biochemical or genetic attempts to demonstrate a role in this pathway have been unsuccessful (Chistoserdova & Lidstrom, 1997; Harms et al., 1996; Ras et al., 1991). Also, studies in P. denitrificans have demonstrated that the primary electron acceptor of MxaFI is cyt c₅₅₁ and not cyt c₅₅₃ while in M. extorquens cyt c₁ directly accepts electrons from methanol dehydrogenase (Anthony, 1992; Long & Anthony, 1991). While we cannot distinguish whether CycB is a direct electron acceptor from XoxF or an intermediate carrier of electrons to the membrane, we have shown that this protein is required by R. sphaeroides when methanol is oxidized to formaldehyde. The intergenic distance between R. sphaeroides cycB and adjacent

Fig. 3. Phylogenetic analysis of XoxF orthologues. Amino acid sequence identities among c₂-proteobacteria XoxF homologues are represented. The phylogenetic tree shows all three wild-type strains of R. sphaeroides grouping in one of two clades based on evolutionary distance. This analysis was generated with MEGA (Kumar et al., 2004), and bootstrap values above 50% from 500 replicates are shown. Scale bar, 0.02 substitutions per site.
ORFs (RSP2580 and RSP2581) combined with computational analysis of global gene-expression studies suggests that these three genes might be co-transcribed (Y. Dufour, personal communication). Thus, our findings could also reflect the effect of the cycB mutation on the expression of RSP2580 and RSP2581. If this was the case, then it would also provide the first evidence for a specific role of these uncharacterized proteins in formaldehyde metabolism.

Our data predict that one or more class I cyt proteins, isocyt c2 or cyt c2 is involved in electron transport from the periplasmic site of formaldehyde formation to the membrane. We propose that one or both of the class I cyt cytochromes transfers electrons to the membrane, especially since previous studies have shown that each cyt c2 isoform can transfer electrons to reaction centre (RC) complexes (Fig. 1b) (Rott et al., 1993).

Purified Gfa of P. denitrificans catalyses the condensation of formaldehyde and glutathione to generate the hydroxymethylGSH adduct used as a substrate by GSH-FDH (Goenrich et al., 2002). We conclude that R. sphaeroides does not absolutely require Gfa for formaldehyde activation because cells lacking Gfa (SW102) were capable of electron transfer from methanol to O2 and able to use methanol as a sole photosynthetic carbon source. Cells that lack GSH-FDH accumulate formaldehyde and are killed by methanol (Barber et al., 1996; Rott et al., 1993). Thus, the properties of SW102 suggest that formaldehyde can spontaneously condense with GSH at a sufficient rate in vivo to prevent accumulation of formaldehyde. In M. extorquens the formaldehyde activating enzyme (Fae) catalyses the condensation of formaldehyde and tetrahydromethanopterin (Vorholt et al., 2000). Unlike Gfa of R. sphaeroides, Fae is necessary for growth on methanol (Vorholt et al., 2000).

In summary, these experiments have identified additional proteins that are required by cells to generate formaldehyde from methylated compounds and to participate in other steps of the metabolism of this compound. Our results indicate that formaldehyde metabolism by R. sphaeroides requires XoxF, PQQ and periplasmic c-type cyt proteins, including CycB, isocyt c2 or cyt c2. However, Gfa is not absolutely required by R. sphaeroides to condense formaldehyde with glutathione. Based on these findings, we predict that the PQQ-dependent dehydrogenase XoxF oxidizes methanol to formaldehyde, which stimulates electron transfer to CycB and isocyt c2 and then onto membrane-associated electron acceptors (Fig. 1b). These findings make specific predictions for further analysis of this pathway in R. sphaeroides and other bacteria which contain a similar putative formaldehyde gene cluster.

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